Running title: Eukaryotic FeSOD crystal structure

The Crystal Structure of an Eukaryotic Iron Superoxide Dismutase suggests Intersubunit Cooperation during Catalysis

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Abstract

Superoxide dismutases (SODs) are a family of metalloenzymes that catalyze the dismutation of superoxide anion radicals into molecular oxygen and hydrogen peroxide. Iron superoxide dismutases (FeSODs) are only expressed in some prokaryotes and plants. A new and highly active FeSOD with an unusual subcellular localization has recently been isolated from the plant *Vigna unguiculata* (cowpea). This protein functions as a homodimer and, in contrast to the other members of the SOD family, is localized to the cytosol. The crystal structure of the recombinant enzyme has been solved and the model refined to 1.97 Å resolution. The superoxide anion binding site is located in a cleft close to the dimer interface. The coordination geometry of the Fe site is a distorted trigonal bipyramidal arrangement, whose axial ligands are His43 and a solvent molecule, and whose in-plane ligands are His95, Asp195 and His199. A comparison of the structural features of cowpea FeSOD with those of homologous SODs reveals subtle differences in regard to the metal-protein interactions and confirms the existence of two regions that may control the traffic of substrate and product: one located near the Fe binding site and another in the dimer interface. The evolutionary conservation of reciprocal interactions of both monomers in neighbouring active sites suggests possible subunit cooperation during catalysis.

Keywords: *antioxidants, iron superoxide dismutase, manganese superxoxide dismutase, X -ray crystallography, protein-protein interaction.*

Abbreviations

SOD, superoxide dismutase; PDB, Protein Data Bank; r.m.s., root-mean-square; ROS, reactive oxygen species; Tm, transition temperature; CuZnSOD, copper zinc superoxide dismutase; FeSOD, iron superoxide dismutase; Vu_FeSOD, *Vigna unguiculata* iron superoxide dismutase; Ec_FeSOD, *Escherichia coli* iron superoxide dismutase; Po_FeSOD, *Pseudomonas ovalis* iron superoxide dismutase; Mt_FeSOD, *Methanobacterium thermoautotrophicum* iron superoxide dismutase; Ss_FeSOD, *Sulfolobus solfataricus* iron superoxide dismutase; MnSOD, manganese superoxide dismutase; Hu_MnSOD, *Homo sapiens* manganese superoxide dismutase; Ec_MnSOD *E. coli* manganese superoxide dismutase.

Introduction

A problem shared by all aerobic organisms is that between 1 and 3% of the oxygen consumed by respiration is partially reduced to some type of reactive oxygen species (ROS), such as the superoxide radical, hydrogen peroxide, and the hydroxyl radical. ROS can oxidize DNA, proteins and lipids, leading ultimately to cell death. Superoxide radicals exhibit only moderate reactivity but can be the precursors of highly oxidizing hydroxyl radicals. SODs catalyze the dismutation of superoxide radicals to oxygen and hydrogen peroxide and thus constitute a major antioxidant defense mechanism. They are present in virtually all aerobes and in several anaerobes. There are three known classes of plant SODs, each coordinating with a different metal cofactor. CuZnSODs are found in the cytosol, chloroplasts, nucleus and apoplast (Kanematsu 1991), MnSODs are observed in mitochondria and peroxisomes (Fridovich 1995) and FeSODs are present in chloroplasts (Kliebenstein 1998). SODs catalyze a two step reaction in which superoxide radicals are dismutated into hydrogen peroxide, while a metal cofactor cycles between a reduced and oxidized forms (Halliwell 1999).

The structures of several SODs of each type have already been reported (six FeSODs, three MnSODs and seven for CuZnSODs). Sequence and structure comparisons show that the MnSOD and FeSOD groups are closely related to each other, whereas the CuZnSOD appear to have evolved independently. Structurally, MnSOD and FeSOD appear to be variants of the same enzyme (Stallings 1984). Both contain an α/β fold which differs from the Greek key β-barrel of CuZnSOD (Tainer 1982). MnSOD and FeSOD are typically observed to be homodimers or homotetramers. Each 200 residues monomer is bound to a metal ion. The active sites of these enzymes are specific for their respective metal ions and for the superoxide anion. They exhibit a conserved structure that consists of a group of metal-binding residues enclosed by a shell of residues.

Although both enzymes have the ability to bind either Mn or Fe, the replacement of the corresponding metal ion in the native SOD decreases enzyme activity (Ose 1976) (Yamakura 1980), a result that is probably due to inappropriate redox potentials (Vance 1998) (Brock 1977).

In this report we present for the first time the structure of a recombinant eukaryotic FeSOD from the plant *Vigna unguiculata* (cowpea). This type of protein, when present in an eukariotic organism, has only been located in the chloroplasts of plants. To our knowledge this is the first FeSOD that has been clearly observed in the cytosol (Moran 2003). Therefore, contrary to the widely held view, FeSODs in plants are not only restricted to the chloroplasts, but also probably constitute a defensive mechanism against oxidative stress associated with senescence. Our model contributes to a deeper understanding of this family of enzymes providing structural data for the most evolved member of this important family of proteins. A structural comparison between cowpea FeSOD and other SODs from distinct organisms belonging to different kingdoms confirms the existence of regions, close to the active site that contain key residues that have essential functions during enzyme catalysis. Furthermore the crystal structure corroborates the presence of interactions between residues of both monomers that have been conserved over evolutionary time and that most likely play an critical role during enzyme catalysis.

Results

Overall structure

Protein characterization and crystallization have previously been reported (Moran 2003) (Muñoz 2003) (see supplementary material). The crystal structure of cowpea iron

superoxide dismutase (Vu_FeSOD), an iron-containing protein with 238 amino acid residues, was solved by molecular replacement and refined to a crystallographic R-factor of 14.8% and R-free of 19.2% (Table I). The crystal belongs to the monoclinic space group C2 and has one monomer per asymmetric unit (Muñoz 2003) (Table I). Vu_FeSOD overall fold is an α/β fold (Figure 1a). There was no electron density evident to model the previous first thirteen residues of the amino acid sequence so that the model starts at residue Lys14 (Figure 1a). The monomer consists of two structural domains: a N-terminal helical domain composed of two long antiparallel α helices, which are separated by a small α-helix and a C-terminal α/β domain, which contains a central three-stranded antiparallel β sheet and four α helices (Figure 1a). The first fifteen amino acid residues at the N-terminus form an extended region that packs antiparallel to helix α1. This helix is slightly kinked around the conserved Lys46 (Edwards 1998). The helix α1 is connected by a turn to helix α2 forming a helical hairpin structure. The residues (Gly-Thr) at the tip of the helical hairpin could not be built into the electron density map. The overall B-factors of the modelled residues at the extremes of this loop (43 and 47 $Å^2$) are higher than the average B-factor (Table I). The short helix α2 connects the two long α1 and α3 helices. The helix α2 is also present in the structures of the bacterial FeSOD from *Escherichia coli* (Ec_FeSOD) and *Pseudomonas ovalis* (Po_FeSOD). This helix is substituted by a long loop that extends towards the solvent in the structures of Archaea *Metanobacterium thermoautothrophicum* (Mt_FeSOD), *Sulfolobus solfataricus* (Ss_FeSOD), and in the human MnSOD (Hu_MnSOD) (Figure 1b). The C-terminal domain is an α/β type fold and consists of a three-stranded antiparallel β-sheet with helices α4, α5, α6 and α7 on one side. A large loop exposed to the solvent joins strands β1 and β2 (Figure 1a). The majority of this loop is absent in other Fe and MnSODs (Figure 1b). In our model, the

loop has a gap comprising nine residues, from Asp155 to Ala165, in which no clear electron density was present to model them. Again, the overall B-factor at the two edges of the gap (49Å^2 and 60 Å^2) are above the average value of the structure (Table I). The metal binding site is located at the interface between the N- and C-terminal domains. The helices α2 and α3 contribute to the binding site with two residues, His43 and His95, whereas the β3 strand and the subsequent loop supply the other two metal ligands, Asp195 and His199.

The active site geometry

The structure and active site geometry are well defined by the electron density map (Figure 2). Although the position of the metal in the active site and the residues nearby are similar to other SODs, several differences in distances and amino acid positions are noteworthy. The Fe is pentacoordinated by four residues (His43, His95, Asp195 and His199) and a solvent molecule that has been proposed to be a hydroxide ion (Figure 2, 3) (Stallings 1991). The five ligands form a spatially arranged trigonal bipyramide around, the iron atom (Stallings et al. 1985). The position of the Fe defines a cavity inside the protein (Figures 3) which is hidden and protected by four additional residues, His47, Tyr51, Gln91 and Trp197. These four residues are involved in control of the transit of substrate and product (Maliekal et al. 2002). Figure 3 illustrates the position of these residues, which form a secondary shell that covers the entrance to the active site. The three aromatic residues are conserved among FeSODs, with the exception of Ss_FeSOD (Figure 1b), in which a phenylalanine is substituted for a tryptophan. Although the glutamine residue and its side chain conformation, which points to the active site, is conserved in Vu_FeSOD, Po_FeSOD and Ec_FeSOD, a histidine can be found in its place in the structures of the Archaea FeSODs (Mt_FeSOD and Ss_FeSOD)

(Figures 1b). The amide group of the histidine side chain occupies a position similar to the glutamine in all FeSOD structures (data not shown). This conserved arrangement suggests that the amide group, which has conserved its position in the active site of different SODs, not only performs an important role during catalysis (Yikilmaz et al. 2002), but may also be involved in metal specificity (Hunter et al. 2002).

The structure of the Hu_MnSOD active site reveals features similar to those of the Archaea Ss_FeSOD and Mt_FeSOD enzymes (Figure 1b). The main difference is due to substitution by an alanine of the tryptophan in the group of residues surrounding the active site. Even though the glutamine is also present, the side chain distance to the proposed hydroxide ion is shorter (2.99 Å compared to 3.20 Å in Vu_FeSOD) than in other reported structures, which suggests a stronger interaction. However, its contact with the tyrosine is weaker; there is a distance of more than 4 Å in contrast with 2.90 Å in the case of Vu_FeSOD (Figure3).

Crystal packing and oligomerization

The FeSOD enzymes can be found in homodimeric or homotetrameric form depending on the organism of origin. Although the protein behaves as a dimer in size exclusion chromatograhy (data not shown), our crystal structure has one protein molecule per asymmetric unit with dimensions 45x31x36 Å (Muñoz 2003). However, crystal packing reveals how the molecule generates a firm contact with one of the other protein monomers. These two monomers are related by a 2-fold axis (Figure 4). The packing between the two molecules is tight, indicating that this dimer represents the usual biological oligomerization state of the enzyme, where the active sites are located in close proximity to the dimer interface with a distance of 18.01 Å between the two Fe atoms (Figure 4). A comparison of these two molecules with other dimeric FeSOD

structures revealed a similar arrangement of the two monomers (Stallings et al. 1983) (Ringe et al. 1983). This, together with the buried surface (1100 $Å^2$) (Nicholls et al. 1991) between the protomers, strongly supports the idea that the dimer represents the biological unit.

From the interface of the two molecules, the solvent region can be divided in two areas, one between the monomers that generates a channel filled with solvent molecules, and a second around each monomer cavity, where the Fe atom is located. Each cavity leads to the channel giving the appearance of a funnel at each site. Figure 5 (see supplementary material) shows the contacts of all conserved residues involved in the formation of the solvent filled channel. The separation between the two molecules ranges between 2.7 and 2.9 Å. A close contact is found at the entrance of the channel, where both monomers interact by hydrogen bonds with their respective Ser142 (2.89 Å) and a water molecule. Another important interaction can be observed between the Glu198-OE2 of each monomer and the His199-ND1 of both catalytic centres (2.73 Å), which involves three additional water molecules. The interaction between Tyr202 from one monomer with His47 from the other (Tyr202-OH to His47-NE2, 2.58 Å) also merits attention. These interactions are conserved in other FeSOD and MnSOD structures, and the hydrogen bond between His47 and Tyr202 seems to be involved in the enzymatic mechanism of Ec_MnSOD (Edwards et al. 2001). The position of the residues and water molecules involved in the channel and cavity arrangement is basically identical in the structures of Vu_FeSOD, Ec_FeSOD, Po_FeSOD and Ss_FeSOD, all of which form a dimer (Figure 1b). In the case of Mt_FeSOD and Hu_MnSOD, the biological unit is a tetramer arranged as a couple of dimers. In this case the residues in the active site and surrondings from one monomer conserve the interactions mentioned above with one of

the neighbouring monomers, but not with the other two. This implies that dimeric interactions have been evolutionarily conserved (Figure 1b), and suggests an active role for the residues of one monomer in the active center of the other during catalysis. Thus, the subunits of the tetrameric FeSODs and MnSODs should perform the enzymatic reaction in a pairwise form.

Discussion

We present the structure of the first eukaryotic FeSOD from cowpea. This enzyme is the first one in its family found in the cytosol (Moran 2003). This implicates Vu_FeSOD in cellular mechanisms of free-radical elimination other than those related to photosynthetically produced superoxide. The structure has been solved to 1.97 Å, which allows us to describe the structural characteristics of this new enzyme and compare them with other reported structures.

A close examination suggests that the simpliest pathway for the superoxide radical to reach the active site is controlled by the Gln91, Tyr51, His47 and Trp197, the residues which build a shell around the metal center (Figure 3) (Whittaker and Whittaker 1997). Hence, the superoxide radical should move through the cavity, interact with these residues, and ultimately be guided to the active site where dismutation takes place. The importance of these residues has been highlighted before (Maliekal et al. 2002) (Jackson et al. 2002) (Yikilmaz et al. 2002). The task of Trp197 is probably essential for the function of Gln91, His47 and Tyr51, since it covers the upper part of the cavity entrance and thus provides an appropriate environment for catalysis. Trp144, which is located near the active site, is also found in all the FeSOD and MnSOD structures (Figures 1b and 3). The positioning of both tryptophans implies little room for alternative

conformations at the active site (Figure 3). The shape and properties of the other FeSOD and MnSOD are identical around most of this group of residues on the side facing the binding cleft. Therefore, although subtle differences in the mechanisms have been reported (Jackson et al. 2002), the driving of the susbtrate probably occurs in a manner common to all of them.

However, it is still not clear how protons are delivered to the substrate in the second step of the enzymatic reaction. The possible existence of several pathways to the active site has been considered before (Edwards et al. 2001). One hypothetical candidate for this role is Tyr51, which may be involved in the direct release and transfer of protons from water molecules found outside the active pocket (Lah 1995). A second candidate may be the conserved Tyr202 from the neighbouring monomer. Both the water molecules and the residues themselves, which are properly positioned to deliver protons to the substrate via these tyrosines, are conserved among most of the known SOD structures. As a result, this hypothesis would imply the requirement of a dimer in order to supply the conserved Tyr202 involved in the enzymatic reaction.

How the oligomerization state of the enzyme relates to the catalytic mechanism is a question that has not been fully answered yet. We can confirm that the residues located in the surroundings of the channel are similar in all the FeSOD and MnSOD structures and that these residues build a similar network of interactions (Figure 1b, 5). The water molecules at the monomers interface are most likely an important factor for the supply of protons to both catalytic sites. Furthermore, important contacts between the subunits in the dimer have been conserved (Stallings et al. 1983) (Ringe et al. 1983) (Edwards et al. 2001). A view of the channel indicates a crucial role for several interactions (Figure

5). As mentioned before, Tyr202 from one monomer might participate in the catalysis of the neighboring active site through its interaction with His47. This histidine is located in the shell of residues around the Fe binding site (Figure 3). The disruption of this interaction reduces the superoxide dismutase activity to 30-40% in Ec_MnSOD (Edwards et al. 2001). Another contact of one monomer with the active site of the other involves a hydrogen bond between Glu198 and His199 (Figure 5). This interaction is of utmost importance to preserve the dimer formation in Ec_MnSOD (Edwards 1998).

The conservation of these residues from Archaea to Eukarya and their interactions argue in favour of their important role in enzyme stability and function (Figure 1b). In addition, a close analysis of the oligomerization state of the different SODs reveals not only a similar organization among the dimeric SODs (superpositions: Vu_FeSOD-Po_FeSOD 0.862 Å r.m.s.d for 370 Cα; Vu_FeSOD-Ec_FeSOD 0.827 Å r.m.s.d for 372 Cα and Vu_FeSOD-Ss_FeSOD 1.18 Å r.m.s.d for 332 Cα), but also the arrangement of the tetrameric SODs as a dimer of dimers (Vu_FeSOD-Mt_FeSOD, 1.18 Å r.m.s.d for 349 Cα and Vu_FeSOD-Hu_MnSOD, 1.22 Å r.m.s.d for 351 Cα). In the case of the tetrameric arrangement, the key interactions are conserved in a pairwise form, including not only the distances among the key residues and the water molecules in the channel, but also the distance between the metal active sites, which is around 18 Å in all of them.

Considering the absence of monomeric SODs in nature together with all the previous information, there is a strong indication that the dimer is the minimal catalytically active form of this type of enzyme. This would imply the possibility of intersubunit cooperation during catalysis. Therefore, based on the analysis of the reported

structures, the tetrameric SODs should function as a couple of dimers that undergo catalysis in an independent manner.

Materials and methods

Protein purification, crystallization and data collection

Preparation and characterization of Vu_FeSOD have previously been described (Moran 2003) (see supplementary material). Crystallization experiments and data collection have been reported by Muñoz et al. (Muñoz 2003). Images were processed and scaled with the HKL program (Otwinowski 1997) and programs of the CCP4 package (Collaborative Computational Project 1994). Statistics for the crystallographic data are summarized in Table I.

Structure solution

The structure was solved using the molecular replacement method as implemented in the program EPMR (Kissinger 1999). The search model was based on an alignment of the amino acid sequence of the Vu_FeSOD with that of Po_FeSOD, obtained using the program CLUSTALW (Higgins D. 1994) and finally it was built by modification of the model of Po_FeSOD found in the Protein Data Bank (Benson 2000) (entry 1DT0). The correct solution was the highest peak in both rotation and translation searches including data between 15.0 and 4.0 Å resolution, with a final correlation coefficient of 0.522. A 2Fo-Fc map showed clear and contiguous electron density for the peptide backbone and for many of the side-chains of the protein.

Model building, refinement and analysis of the final model

Five percent of the reflections of the data set were set aside for free R-factor calculations during refinement (Brünger 1992). Positions where the sequence differed were designated alanine and glycine. The electron density map was calculated using only the working set of reflections and the model was rebuilt where the electron density supported changes. When clear density was observed in place of the side-chain expected in Vu_FeSOD, the model was mutated accordingly and the side-chain fitted into the density. The resulting model was then refined against the 1.97 Å data set using CNS (Brünger 1998) for the first round of the refinement, including a rigid body minimization followed by simulated annealing (Cartesian starting at 5000 K). The R-factor and R-free values after this first cycle were 0.331 and 0.346, respectively. Further rounds of model mutation/rebuilding were performed using the program O (Jones 1991). Refinement proceeded with the program REFMAC5 (Murshudov 1999) including a rigid-body refinement as the first step. The data were anisotropic, and the most successful refinement strategy made use of Babinet's bulk solvent correction (Moews 1975) combined with overall anisotropic scaling and individual anisotropic temperature factor refinement using maximum likelihood as implemented in REFMAC5. Several rounds of rebuilding using the program O and the placement of the iron atom and the water molecules into the electron density, resulted in the final model. The statistics after crystallographic refinement of this model are summarized in Table I. All the structure superpositions were performed with the use of the program O lsq routine (Jones 1991). Coordinates and the corresponding structure factor data, have been deposited in the RCSB Protein Data Bank (Benson 2000) with entry code 1unf.

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Table I. Data collection and refinement statistics

Data collection[a]	
Environment	130mm MarCCD, ESRF, beamline BM14S
Wavelength	1.033 Å
Cell dimensions (Å, °), space group C2	a=81.98, b=48.16, c=63.67, β=119.76
Resolution (Å)	55.3-1.97 (2.017-1.97)
Unique reflections	14662
Average multiplicity	3.4 (3.2)
Completeness	99.6 (99.9)
R_{merge}[b]	0.07 (0.34)
$\langle I/\sigma I \rangle$	8.3(2.9)
Refinement	
Number of reflections (completeness, %)	14662 (99.9)
Resolution range (Å)	55.3-1.97
R-factor / R-free (%)	14.8/19.2
Number of protein atoms (Average B, $Å^2$)[c]	1724 (22.67)
Number of water molecules (Average B, $Å^2$)[c]	184 (29.33)
Number of ligand atoms (Average B, $Å^2$)	1/11.18
r.m.s bond length (Å)	0.014
r.m.s. bond angle (°)	1.585
Ramachandran plot outliers (number)[d]	0

[a] Values in the highest resolution shell are given in parentheses.
[b] $R_{merge} = \Sigma_\eta \Sigma_\iota | I_{\eta,\iota} - \langle I_\eta \rangle | / \Sigma_\eta \Sigma_\iota | I_{\eta,\iota}|$
[c] Calculated using MOLEMAN.
[d] Calculated using PROCHECK.

Figure legends

Figure 1. a) Cα ribbon representation of the FeSOD structure of *Vigna unguiculata*. Helical segments are shown in blue, β–strands in purple and loop regions in grey. Helices are labelled from α1 to α7 and β–strands from β-1 to β-3. The catalytic residues are shown in detail with the solvent molecule in red and the iron atom in green. Residues Lys14 at the N-terminal, Ala238 at the C-terminal and Val59-Thr62, Asp155-Ala165 corresponding to the residues at the two gaps have been numbered for clarity. **b)** Sequence alignment of Hu_MnSOD with FeSOD from plants (Vu_FeSOD), bacteria (Ec_FeSOD and Po_FeSOD) and Archaea (Mt_FeSOD and Ss_FeSOD). All the structures were pairwise aligned based on the three dimensional structure of FeSOD from Vu_FeSOD. The yellow boxes indicate the residues that form the catalytic site in all the enzymes, magenta boxes indicate the residues that compose the shell opposite to the catalytic site, green boxes indicate the position of the residues that are involved in the interface. Figure 1a was prepared using Molscript (Kraulis 1991) and POV-Ray (http://www.povray.org). Figure 1b was prepared using ALSCRIPT (Barton 1993).

Figure 2. FeSOD active site and geometry. View of a 2(|Fo|-|Fc|) omit map at 1.97 Å contoured at 1 σ showing the residues and the solvent molecule involved in the binding of the catalytic iron. The omit map was calculated with the program OMIT in the CCP4 package. Figures 3, 4 and 5 were prepared using O (Jones 1991), and Molray (Harris and Jones 2001).

Figure 3. Cα ribbon diagram showing a detailed view of the amino acids that form the active site in Vu_FeSOD. The catalytic residues, His 43, 95, 199 and Asp 195, are coloured in yellow, while the second shell amino acids, His47, Tyr51, Gln91, Trp144

and Trp197, in magenta. The solvent molecules, which form part of the secondary shell, opposite to the active site are coloured purple. The hydrogen bond network between the shell forming residues is shown as gold bubbled lines.

Figure 4. Interaction between the FeSOD monomers in the crystal. The depicted dimer is the biological unit in vivo. Each monomer is coloured in blue and gold. The distance between iron atoms is 18.01 Å. The residues involved in iron binding are represented in ball and stick and the iron atom and the water molecule are coloured in green and red, respectively.

Figure 5. View of the channel created between the monomers. Backbone representation in blue and gold respectively. The catalytic residues are coloured in yellow, the shell forming residues in magenta, and the residues of the channel involved in interactions between monomers in green. The water molecules are coloured in pale blue. The interactions between Ser142, Glu198 and Tyr202 of one monomer with Ser142, His199 and His47 of the other monomer are depicted in detail.